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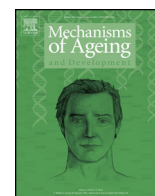
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## Quality control data of physiological and immunological biomarkers measured in serum and plasma



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### ABSTRACT

In two work packages of the MARK-AGE project, 37 immunological and physiological biomarkers were measured in 3637 serum, plasma or blood samples in five batches during a period of 4 years.

The quality of the serum and plasma samples was very good as judged by the low number of biomarker measurements (only 0.2%) that were rejected because of a high hemolysis, ictericia or lipemia of the samples.

Using quality control samples, day-to-day and batch variations were determined. The mean inter-assay variation of the five batches were all below 8%, with an average inter-assay coefficient of variation of all biomarkers of 4.0%. Also the precision of the measurements was very good, because all measurements were between 90% and 115% of the defined target values.

A possible mix-up of samples was determined by comparison of the extreme testosterone levels of men and women. It was concluded that 3% of the sample identification could be mixed-up.

Considering the complex procedure from collection to analysis, including preparation, handling, shipment and storage, of the samples in the MARK-AGE project, both the quality of the samples and the quality of the measurements are very good.

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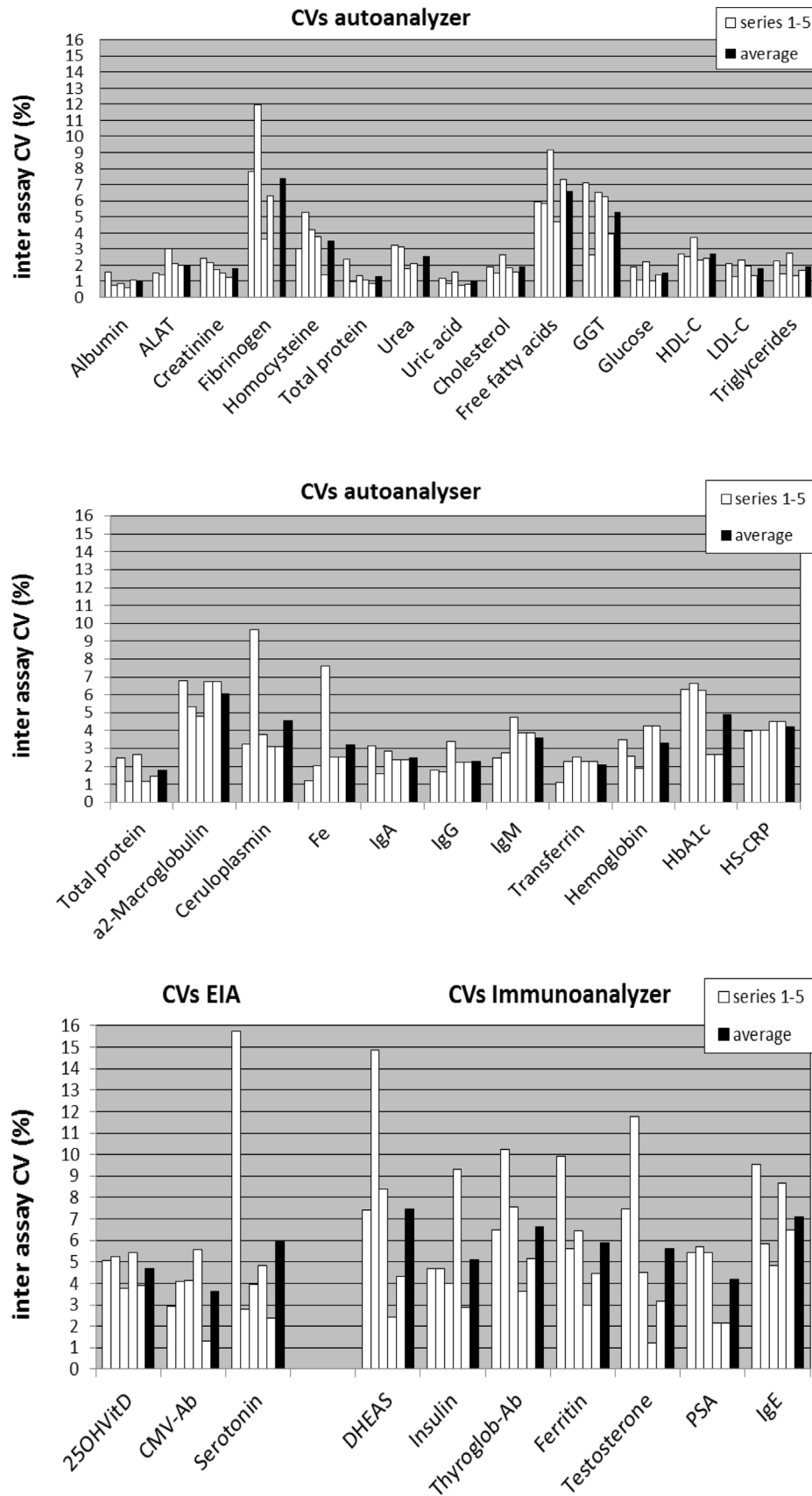
### 1. Introduction

MARK-AGE is a large-scale integrated project supported by the European Commission. The major aim of this project is to conduct a population study in order to identify a set of biomarkers of ageing which, as a combination of parameters with appropriate weighting, would measure biological age better than any marker in isolation.

A total of 37 immunological and physiological biomarkers were measured in 3637 serum, plasma or blood samples at the Cen-

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**Fig.1.** Mean inter-assay CVs (expressed as %) of 36 biomarkers measured in the different series of the MARK-AGE project.

**Table 1**  
List of all physiological and immunological biomarkers, measured by the National Institute for Public Health and the Environment in Bilthoven, the Netherlands and the material and method of analysis.

Biomarker	Abbreviation	Matrix	Method of analysis	Company of assay	Assay product nr
25hydroxy-vitamin D	25OHVitD	EDTA	EIA	IDS	AC57F1
$\alpha$ 2-macroglobulin	AMG	Serum	Autoanal	Dialab	A00505
Alanine aminotransferase	ALT	EDTA	Autoanal	Beckman	476826
Albumin	ALB	EDTA	Autoanal	Beckman	442765
IgG ab against 8 nuclear antigens	ANA-8	Serum	EIA	Dialab	R99419
IgG ab against cytomegalovirus	CMV-ab	Serum	EIA	DRG	EIA-3468
Thyroglobulin autoantibodies	TG-ab	EDTA	Immunoanal	Beckman	A32898
Ceruloplasmin	CER	Serum	Autoanal	Dialab	A00531
Cholesterol	CHOL	Serum	Autoanal	Beckman	467825
Creatinine	CREA	EDTA	Autoanal	Beckman	442760
Dehydroepiandrosterone sulphate	DHEA-S	EDTA	Immunoanal	Beckman	A10826
Total iron	Fe	Serum	Autoanal	Beckman	467910
Ferritin	FER	Serum	Immunoanal	Beckman	33020
Fibrinogen	FIB	EDTA	Autoanal	Dialab	A00517
Free fatty acids	FFA	Serum	Autoanal	Wako	NEFA-HR
$\gamma$ -Glutamyl transferase	GGT	Serum	Autoanal	Beckman	476846
Glucose	GLU	Serum	Autoanal	Beckman	442640
Hemoglobin	Hb	Blood	Autoanal	Beckman	A17836
Glycated hemoglobin	HbA1c	Blood	Autoanal	Beckman	A17836
Homocysteine	HCy	EDTA	Autoanal	Dialab	908520
High-density lipoprotein cholesterol	HDL-C	Serum	Autoanal	Beckman	650207
High-sensitive C-reactive protein	HS-CRP	Li-Hep	Autoanal	Beckman	378020
Immunoglobulin A	IgA	Serum	Autoanal	Beckman	467920
Immunoglobulin E	IgE	Serum	Immunoanal	Beckman	35000
Immunoglobulin G	IgG	Serum	Autoanal	Beckman	467925
Immunoglobulin M	IgM	Serum	Autoanal	Beckman	467930
Insulin	INS	EDTA	immunoanal	Beckman	33410
Low-density lipoprotein cholesterol	LDL-C	Serum	Autoanal	Beckman	969706
Prostate specific antigen	PSA	Serum	Immunoanal	Beckman	37200
Serotonin	SER	Serum	EIA	Biosource	10-0900
Testosterone	TEST	Serum	immunoanal	Beckman	33560
Total protein of serum	s-TP	Serum	Autoanal	Beckman	442740
Total protein of plasma	p-TP	EDTA	Autoanal	Beckman	442740
Transferrin	TFN	Serum	Autoanal	Beckman	467942
Triglycerides	TG	Serum	Autoanal	Beckman	445850
Urea	UREA	EDTA	Autoanal	Beckman	442820
Uric acid	UA	EDTA	Autoanal	Beckman	442785

Abbreviations: EDTA = EDTA-plasma; Li-hep = lithium-heparin plasma; autoanal = autoanalyzer; immunoanal = immunoanalyzer; EIA = enzyme immunoassay.

ter for Health Protection of the Dutch National Institute for Public Health and the Environment within Work package 4: “Immunological markers” and Work package 5: “Clinical chemistry, hormones and markers of metabolism”.

In this paper we present quality control data, day-to-day and batch variations and a possible number of sample exchanges for the whole 4-year period.

## 2. Materials and methods

In the MARK-AGE project in total 3637 samples were distributed amongst the various laboratories from the coordination centre at the University of Hohenheim. In the Center for Health Protection of the Dutch National Institute for Public Health and the Environment in Bilthoven, 37 biomarkers have been measured. The biomarkers were measured in serum, EDTA-plasma, citrate plasma and erythrocytes in 5 batches (1444, 775, 685, 354 and 379 samples) over a 4-year period.

For each biomarker, two reference samples were included in the daily routine. Each day about 100 samples were measured. In addition, each sample was checked for hemolytic (H), icteric (I) and lipemic (L) disturbances by measuring the H-, I- and L-index on the clinical auto-analyzer (LX20-Pro, Beckman-Coulter, Woerden, The Netherlands).

All assays measured on the LX20 autoanalyzer were obtained from Beckman-Coulter, except HCy and AMG (Dialab, Neudorf, Austria), FFA (Wako Diagnostics, Neuss, Germany). Most of the assays on the LX20 were enzymatic assay with a colorimetric end-

point. The assays for AMG, CER, FIB, HbA1c, HDL-C, LDL-C, HS-CRP, IgA, IgG, IgM and transferrin were performed using turbidimetric assays (immunoprecipitation). An extensive description of the LX20 autoanalyzer including the different assays was described by Mikolaenko et al., (2000).

Seven biomarkers have been measured with an immunoanalyzer (Access-2, Beckman-Coulter, Woerden, The Netherlands). These 7 biomarkers were dedicated assays on the Access-2 and obtained from Beckman-Coulter. The Access-2 system is an automated immunoanalyzer based of the principle of chemiluminescence detection using acridinium esters. The assays are performed in a homogenous format without the necessity of pretreatment of the samples. An extensive description of the immunoanalyzer and the dedicated assays have been described by Patterson et al., (1994). Four biomarkers have been measured with enzyme immunoassays (EIA): 25OHVitD (OCTEIA, AC57F1, IDS, Boldon, UK), SER (KAPL10-0900, Biosource, Nivelles, Belgium), CMV-ab (EIA-3468, DRG Diagnostics, Marburg, Germany), ANA-8 (ANA Screen 8 IgG, R99419, Dialab, Neudorf, Austria). Technical information about the biomarkers can be found in Table 1.

## 3. Results

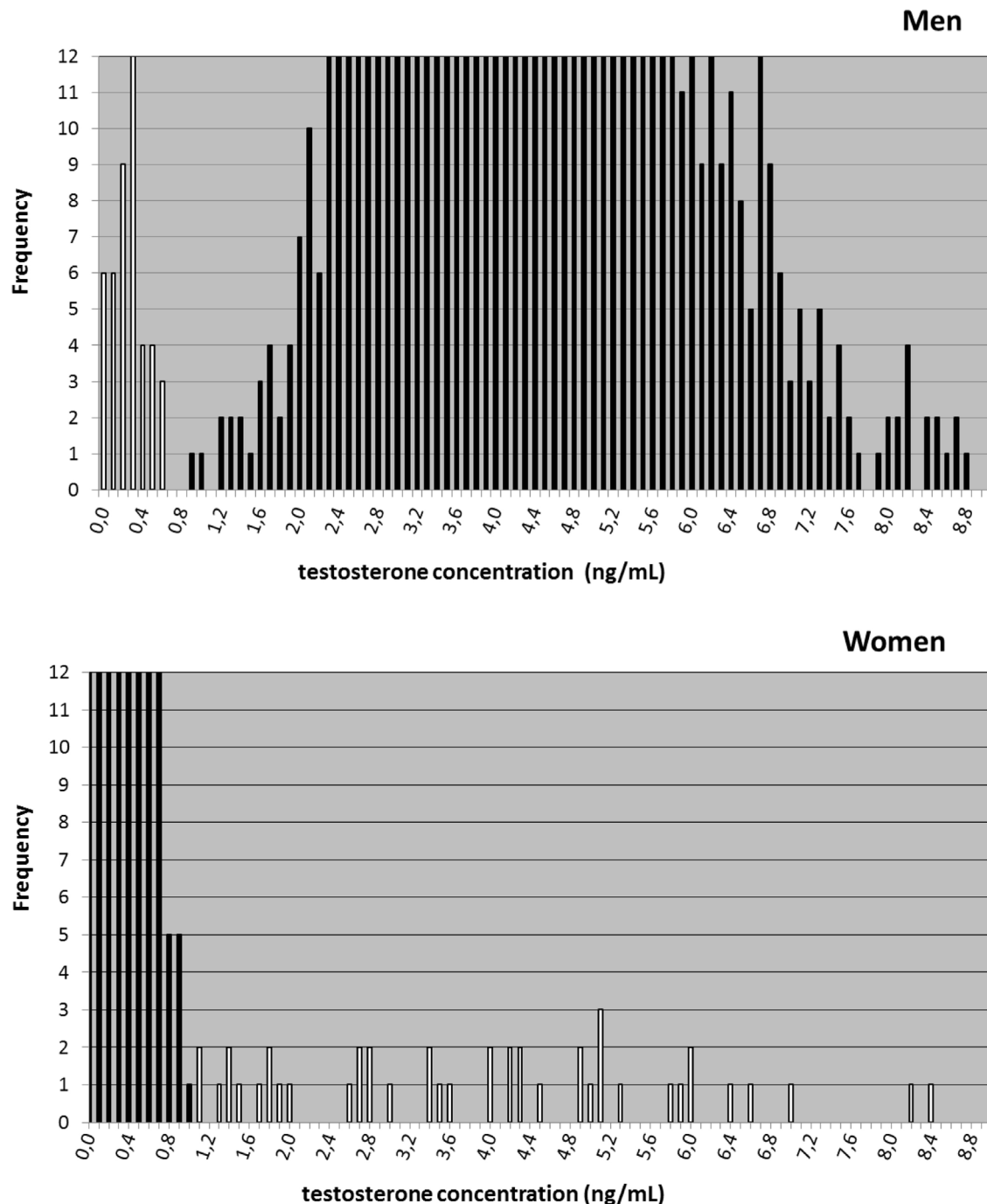
In each serum and plasma sample, a first quality control check was done by the determination of the extent of hemolysis, high levels of bilirubin or lipids, expressed as hemolytic, icteric and lipemic index (H-, I- and L-index), respectively, measured by an

automatic procedure on the clinical auto-analyzer. Each of these three indices is expressed in arbitrary units from 0 to 10. For most of the biomarkers, threshold values are known for each of the three indices (Vermeer et al., 2007). If one of the three indices of the sample is higher than the established threshold values for a particular biomarker, the determined value of the biomarker is probable to deviate from a normal sample condition and therefore the respective biomarker value should be discarded.

For EDTA-plasma the three indices were determined in 3637 samples and compared with the threshold values that were known for 12 biomarkers. The result of this quality check was that of 43,644 biomarker values, only 49 (0.11%) used should be excluded for anal-

ysis (Table 2). For the serum samples, the threshold values of the H-, I-, and L-index were known for 20 biomarkers. In this case, from the 72,740 determinations, 171 (0.24%) should be excluded (Table 3).

Another interesting observation was done from a comparison between the H-, I- and L-indices in serum and EDTA plasma of the same individuals. It appeared that the correlation between the H-indices of serum and EDTA-plasma is rather low (0.11), whereas the correlation of the I- and L-indices between serum and plasma is 0.83 and 0.74, respectively (results not shown). The explanation is that ictericia and lipemia are an inherent property present in the blood of the individual, whereas hemolysis is caused by the preparation of serum and plasma.



**Fig. 2.** Histogram of the testosterone concentrations in all men (upper graph) and women (lower graph). The participants that have extreme testosterone concentrations have been indicated with white bars.

**Table 2**  
The threshold values of the H-, I- and L-indices for the biomarkers that have been measured in serum samples and the corresponding number of data that have been excluded from the database.

Serum	H-index	I-index	L-index	Excluded
s-TP	10	10	3	22
GLU	7	7	9	9
Fe	2	10	10	36
TRF	10	10	10	5
CER	10	10	10	5
GGT	5	10	10	8
CHOL	10	10	10	6
TG	10	3	X	7
HDL-C	10	10	10	5
LDL-C	9	10	10	5
FFA	5	10	10	8
IgG	10	10	10	5
IgA	10	10	10	5
IgM	10	10	6	9
FER	7	5	10	7
IgE	10	8	10	6
PSA	10	10	10	5
TEST	10	8	10	6
AMG	4	8	10	7
ANA-8	10	10	10	5
				Total 171
				0.21%

Samples with a high H-index did not correlate to age or gender of the individuals. We observed however that most of the hemolytic samples originate from one center and that two centers had almost no hemolytic serum samples.

For 26 of the 33 biomarkers measured with the auto-analyzer or the immuno-analyzer, the reference samples had well-defined target values. From the 5 series of measurements 80% of the biomarker measurements were between 95% and 105% of the target values and all measurements were between 90% and 115% of the target values.

In all measuring sessions (usually 100 samples per day), at least one quality control sample was included. In Fig. 1 the inter-assay coefficients of variation (CVs) of one quality control sample (with reference values in the normal physiological range) for each of the five series of measurements have been plotted. The CVs have been expressed as percentage of the standard deviation divided by the mean value). The assay for ANA-8 was only semi-quantitative and therefore no quantitative data of the quality control is available. Of the remaining 36 biomarkers and 180 series of measurements, almost all CVs were below 10%, with the exception of only five measurements. The average CVs, being the means of the five measurement series were all below 7–8%. The average inter-assay CV

**Table 3**  
The threshold values of the H-, I- and L-indices for the biomarkers that have been measured in EDTA-plasma and the corresponding number of data that have been excluded from the database. The “x” in the table for TG means that the Lipemic index in fact is a reflection of the TG concentration in the sample, so no index number can be given.

EDTA-pl	H-index	I-index	L-index	Excluded
p-TP	10	10	3	24
ALB	10	10	10	2
UREA	10	10	10	2
CREA	10	10	10	2
ALT	2	10	9	2
FIB	10	10	10	2
DHEAS	10	10	10	2
INS	10	8	10	2
Tg-ab	10	10	10	2
UA	3	5	10	5
Hcy	10	10	10	2
25OHVifD	10	10	10	2
				Total 49
				0.11%

of all biomarkers was about 4%, whereas the clinical auto-analyzer showed lower CVs than the immuno-analyzer and the ELISAs.

Upon completion of the measurements, gender information was released for 2871 analysed samples, allowing the analysis of testosterone concentration ranges for both men and women. The normal range for testosterone in women and men does not overlap, being 0.06–0.86 ng/mL and 2.69–10.70 ng/mL for women and men, respectively (Kratz et al., 2004). In Fig. 2 histograms are shown of both the testosterone concentrations of men (upper graph) and women (lower graph). We noticed that 44 samples from female participants were in a concentration range that is characteristic for men (white bars), while an identical number of male samples, i.e. 44, had concentrations typically found in women (white bars). Furthermore it is observed that 18 of 19 women (94%) with PSA > 1 ng/ml are among the 44 women with most extreme testosterone concentrations. Together, these results suggest that 88 samples were exchanged between genders, being 3%.

#### 4. Conclusions

In the recruiter centers of the MARK-AGE project, the sampling was performed according a very strict protocol (see Moreno-Villanueva, Capri et al., this issue). As a result, the quality of the serum and plasma samples was very good. This was judged by the three indices that were determined on the clinical auto-analyzer. Both the hemolytic, icteric and lipemic index show in general very low levels, indicating that only a small part of the biomarkers could not be used for the final analysis. From the in total 116,384 biomarkers measured, only 130 (0.2%) cannot be used because of a too high index. This percentage is comparable with the finding of another European study on aging (see Boffetta et al., 2014). By correlation analysis it appeared that especially the hemolytic index determines the quality of the sample preparation and not the icteric or lipemic index.

The quality control of the analyses was performed by including control samples at the beginning and end of each analysis session. The average inter-assay variation of all biomarkers was 4.0%. As is shown in Fig. 1, the CVs of the immunochemical methods (EIA and immunoanalyzer) are somewhat higher than the CVs of the colorimetric assays of the autoanalyzer, because of the more complicated assays of the immunochemical methods.

Sample exchanges or database errors are difficult to avoid in complex large-scale projects. Sample or data exchange could have occurred at several stages, including recruiting, storage and relabeling of samples, samples analysis including reporting and data storage, all done at different sites (see Bürkle et al.; Moreno-Villanueva, Capri et al.; Capri, Moreno-Villanueva et al.; Baur et al., this issue). One possible check of sample mix-up is the correlation between the participant's gender and the testosterone levels. An equal number of designated male and female samples showed testosterone levels in the range of the opposite gender, with almost half of the designated samples from females having the highest PSA values measured in this group. Possibly, these samples (3% of total) got exchanged anywhere in the chain from sample collection to data analysis. As potential sample exchanges within sexes go unnoticed in this analysis bases on sex differences, the estimated exchange rate for all sample sets may be twice as high.

In the MARK-AGE project, both the quality of the serum and plasma samples and the quality of the biomarkers measurements are excellent, considering the complicated procedures in this large-scale project. The quality includes preparation, handling, shipments, (re)numbering, storage conditions, quality control procedures and measurements of the blood samples.

## Conflict of interest

All authors approved the final manuscript and none of the authors had any personal or financial conflict of interest.

## Acknowledgements

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